Sheep carrying pathogenic Yersinia enterocolitica bioserotypes 2/O:9 and 5/O:3 in the feces at slaughter

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Abstract: belonging to biotypes 1B and 2-5. Pathogenic strains of biotypes 2-4 carrying the ail virulence gene have frequently been isolated from domestic pigs at slaughter. In sheep, mostly non-pathogenic biotype 1A strains have been reported. In our study, the prevalence of ail-positive Y. enterocolitica was studied by PCR and culturing in 406 young sheep (<1year of age) and 139 older sheep at slaughter in Finland. When using PCR, the detection rate was 11% (45/406) in young sheep originating from 11 (18%) farms. Surprisingly, Y. enterocolitica belonging to bioserotypes 2/O:9 and 5/O:3, carrying both chromosomal and plasmid-borne virulence genes, were isolated from the fecal samples of 10 (2%) and 23 (4%) sheep, respectively. All isolates of bioserotypes 2/O:9 (19 isolates) and 5/O:3 (53 isolates) carried the chromosomal virulence genes ail, inv, ystA, and myfA, and almost all isolates (71/72) also carried the virulence genes virF and yadA located on the virulence plasmid. The isolates showed high susceptibility to tested antimicrobials and low genetic diversity by PFGE. Y. enterocolitica bioserotype 5/O:3 is a very rare bioserotype, and has earlier only sporadically been reported in European wildlife and in sheep in Australia and New Zealand. Bioserotype 2/O:9 is a common bioserotype found in humans with yersiniosis, and has sporadically been isolated in wild and domestic animals.

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Sheep carrying pathogenic *Yersinia enterocolitica* bioserotypes 2/O:9 and 5/O:3 in the feces at slaughter

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Running head: *Yersinia enterocolitica* in sheep

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Abstract

Yersina entrocolitica is a heterogeneous species including non-pathogenic strains belonging to biotype 1A and pathogenic strains belonging to biotypes 2 to 5. Pathogenic strains belonging to biotypes 2 to 4, especially biotype 4, have frequently been isolated from domestic pigs at slaughter. Only few studies have been conducted on the situation in sheep. In this study, the prevalence of *ail*-positive *Y. enterocolitica* was studied by PCR and culturing in 406 young sheep (<1 year) and 139 older sheep at slaughter in Finland. The detection rate was 11% (45/406) in young sheep originating from 11 (18%) farms by PCR. Surprisingly, *Y. enterocolitica* belonging to bioserotypes 2/O:9 and 5/O:3 carrying both chromosomal and plasmid-borne virulence genes were isolated from the fecal samples of 10 (2%) and 23 (4%) sheep, respectively. All isolates of bioserotypes 2/O:9 (19 isolates) and 5/O:3 (53 isolates) carried the chromosomal virulence genes *ail*, *inv*, *yst*A and *myf*A, and almost all isolates (71/72) carried also the virulence genes *vir*F and *yad*A encoded on the virulence plasmid. The isolates showed high susceptibility to tested antimicrobials and low genetic diversity by PFGE. *Y. enterocolitica* bioserotype 5/O:3 is a very rare bioserotype and was reported earlier only sporadically in wildlife in Europe and in sheep in Australia and New Zealand. Bioserotype 2/O:9 is a common bioserotype found in humans with yersiniosis and has sporadically been isolated in wild and domestic animals.

Key words: *Yersinia enterocolitica*, biotype, sheep, feces

INTRODUCTION

Yersinia enterocolitica and *Yersinia pseudotuberculosis* strains carrying the virulence plasmid (pYV) can cause enteritis and symptoms mimicking appendicitis due to mesenteric lymphangitis. Enteric yersiniosis is a common disease in Europe with around 6000 reported cases (2 cases/100,000 inhabitants) in 2014 (EFSA, 2015). The highest country-specific rates were observed in Finland (11 cases/100,000 inhabitants) and Denmark (8 cases/100,000 inhabitants). *Y. enterocolitica* was the most common species isolated from patients and the most common serotypes were O:3 (83%), O:9 (14%) and O:5,27 (2%).

Y. enterocolitica has been isolated from several animal species including farm animals; however, fattening pigs are so far the only animal species frequently carrying pYV-positive strains in the tonsils and excreting this pathogen also in the feces (Fredriksson-Ahomaa, 2015). The pig strains are typically belonging to serotype O:3 and biotype 4, which is also the biotype most commonly associated with human yersiniosis in Europe (EFSA, 2015). Serotype O:3 strains belonging to biotype 3 has frequently been isolated in Asia from both humans and animals, especially from pigs and dogs (Liang et al., 2015). Bioserotype 3/O:3 and 5/O:3 strains have been isolated from chinchilla and hares in Europe. It has been suggested that ruminants, especially cattle, may play an important role as a reservoir for bioserotype 2/O:9 (Fredriksson-Ahomaa, 2015).

The presence of enteropathogenic *Yersinia* in small ruminants has to a limited extent been studied. Mostly non-pathogenic *Y. enterocolitica* strains of biotype 1A have been detected in Europe (Milnes et al., 2008; Söderqvist et al., 2012). However, quite recently,. *Y. enterocolitica* serotype O:9 was isolated in Sweden from sheep. In Australia, *Y.*

pseudotuberculosis serotype O:3 and *Y. enterocolitica* serotype O:2,3 have been isolated from asymptomatic sheep (Slee & Skilbeck, 1992). Young animals were shown to excrete *Y. enterocolitica* in feces typically for 3 to 16 weeks but sometimes even up to 29 weeks.

In Finland, sheep farming is semi intensive and small-scale; the average number of ewes on the farms is only 50 and one third of the farms are organic farms. However, the number of sheep has doubled during the last decade. It has been forecasted that sheep farming continues to grow in the future due to the customers' decision to eat meat products produced in a more ethical and sustainable manner. Enteropathogenic *Yersinia* spp. are public health hazards which may be transmitted via contaminated mutton and, thus, the presence of these pathogens in sheep at slaughter was investigated. The screening was conducted with a real-time PCR approach to increase the detection sensitivity compared to culturing. All PCR-positive samples were also studied with culturing to obtain isolates for further characterization. The biotype, virulence genes and antimicrobial resistance of the *Yersinia* isolates were studied to assess their public health significance and PFGE was carried out to get some information about the genetic diversity of *Yersinia* strains circulation among Finnish sheep.

MATERIALS AND METHODS

Screening of *ail*-positive *Yersinia* spp. in sheep feces by PCR

Fecal samples from 545 sheep originating from 60 farms were collected at slaughter in Finland during 2013 to study the prevalence of *ail*-positive enteropathogenic *Yersinia* spp. in sheep. Most (406/545, 74%) of the animals sampled were under one year old (Table 1). The prevalence of *ail*-positive enteropathogenic *Yersinia* was studied by real-time PCR based on SYBRGreen using the CFX96 system (BioRad, USA) (Joutsen et al., 2012). The DNA was

isolated from the fecal samples by ZR Fecal DNA MiniPrep™ (Nordic BioSite Oy, Helsinki, Finland). Shortly, a 1-g fecal sample with 9 ml of buffered peptone water (BPW, Labema, Kerava, Finland) was mixed with a vortex mixer and incubated at 37°C for 18 to 20 h. The DNA was isolated from 1 ml of the enrichment according to the manufacturer's instructions. Two µl of the DNA was added to 18 µl of mastermix (iQ™ SYBRGreen Supermix, BioRad) containing primers to detect *ail* of *Y. enterocolitica* (Thisted Lambertz et al., 2008a) and *Y. pseudotuberculosis* (Thisted Lambertz et al., 2008b). Additionally, the PCR-positive samples were run separately with *ail* primers for *Y. enterocolitica* and *Y. pseudotuberculosis*. The threshold cycle (Ct) under 38 and a specific melting temperature (Tm) of 79.0 ± 0.5°C indicated a positive result.

Isolation of *Yersinia* from PCR-positive feces samples

PCR-positive homogenates were enriched at 6°C for at least 3 weeks, and then 10 µl of the enrichment was inoculated on cefsulodin-irgasan-novobiosin (CIN) plates (Labema) which were incubated at 28°C for 24-48 h. The colony morphology on CIN plates was studied by stereo-microscope. Up to four colonies with red centers surrounded by a transparent border and of different sizes were picked up from the CIN agar and sub-cultured on blood agar and then tested for the urease enzyme. Urease-positive colonies were identified by API 20E (BioMerieux, France).

Phenotypic characterization of *Yersinia* isolates

All *Yersinia* isolates were inoculated on CHROMagar™ Yersinia plates (Labema). Furthermore, the isolates were biotyped using pyrazinamidase, tween esterase, esculin hydrolysis, indole production, salicin, xylose and trehalose fermentation tests, and the isolates were also serotyped by commercial antisera for antigens O:1,2, O:3, O:5, O:8 and O:9 (Denka

Seiken, Japan) (Joutsen et al., 2013). Presence of the virulence plasmid (pYV) was studied by Congo-red-magnesium-oxalate (CRMOX) agar plates (Tammer-Tutkan Maljat, Tampere, Finland) which were incubated 24 to 48 h at 25 and 37°C. Additionally, susceptibility to 14 antimicrobial agents was tested by broth microdilution using VetMIC™GN-mo panel (Version 4, SVA, Uppsala, Sweden) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008) (Table 3S). The incubation for 18 h at 28°C, which is the optimal growth temperature for both pYV-positive and negative *Yersinia*, was used. The breakpoint set for Enterobacteriaceae or *Escherichia coli* were used according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines version 5.0 (http://www.eucast.org/clinical_breakpoints/). If EUCAST breakpoints were not available for Enterobacteriaceae or *Escherichia coli*, breakpoints for Enterobacteriaceae from CLSI (2014) were used. The control strain was *E. coli* ATCC 25922.

Genotypic characterization of *Yersinia* isolates

The presence of seven virulence genes (*ail*, *yst*A, *yst*B, *inv* and *myf*A in the chromosome and *yad*A and *vir*F on the plasmid) was studied by PCR (Joutsen et al., 2013). Serotypes O:3 and O:9 were confirmed by PCR targeting the *rfb*C and *per* genes, respectively (Fredriksson-Ahomaa et al., 2011). The annealing temperature in all PCR runs was 58°C for all the primers used in this study (Table S1). At least one isolate per sample was characterized with PFGE using *Not*I and *Xho*I enzymes (Fredriksson-Ahomaa et al., 2011).

Table S1 Primers used for real-time PCR based on SYBRGreen to detect *ail*, *yst*A, *yst*B, *inv*, *myf*A, *rfb*C and *per* genes in the chromosome and *yad*A and *vir*F on the virulence plasmid.

Gene	Primers	Product (bp)	Reference

ail-ye	ATGATAACTGGGGAGTAATAGGTTCG CCCAGTAATCCATAAAGGCTAACATAT	163	Thisted Lambertz et al., 2008a
ail-yp	CGT CTG TTA ATG TGT ATG CCG AAG GAA CCT ATC ACT CCC CAG TCA TTA TT	157	Thisted Lambertz et al., 2008b
inv	CTG TGG GGA GAG TGG GGA AGT TTG G GAA CTG CTT GAA TCC CTG AAA ACC G	570	Rasmussen et al., 1994
ystA	ATC GAC ACC AAT AAC CGC TGA G CCA ATC ACT ACT GAC TTC GGC T	79	Thoerner et al., 2003
ystB	GTA CAT TAG GCC AAG AGA CG GCA ACA TAC CTC ACA ACA CC	146	Thoerner et al., 2003
myfA	CAG ATA CAC CTG CCT TCC ATC T CTC GAC ATA TCC CTC AAC ACG C	272	Bhagat & Virdi 2007
rfbC	CGC ATC TGG GAC ACT AAT TCG CCA CGA ATT CCA TCA AAA CCA CC	405	Weynants et al., 1996
per	TGT GCT GAA GCT TTT GGA TCT GAG GCC GAT ACA CCT TGA TT	181	Jacobsen et al., 2005
yadA	TGT TCT CAT CTC CAT ATG C TCC TTT CGC TGC TTC AGC A	203	Fukushima et al., 2003
virF	TCA TGG CAG AAC AGC AGT CAG ACT CAT CTT ACC ATT AAG AAG	590	Wren & Tabaqchali, 1990

RESULTS

Y. enterocolitica carrying the *ail* gene was detected in 45 (8%) feces samples from sheep at slaughter (Table 1). All positive sheep were from animals under 1 year and they originated from 11 (18%) farms. No *Y. pseudotuberculosis* was detected in the fecal samples by PCR.

140 **Table 1** Detection of *ail*-positive *Yersinia enterocolitica* by PCR in 545 sheep originating
141 from 60 farms in Finland 2013.

Number of farms	Number of animals			Number of positive animals		
	All	< 1 year	> 1 year	All	< 1 year	> 1 year
11 (18%)	139	128 (92%)	11 (8%)	45 (32%)	45 (35%)	0
49 (82%)	406	278 (68%)	128 (32%)	0		
60 (100%)	545	406 (74%)	139 (26%)	45 (8%)	45 (11 %)	0

142

143 The detection rate of *ail*-positive *Y. enterocolitica* on the farms varied clearly (Table 2). From
144 most (35/45, 78%) PCR-positive animals, *ail*-positive *Y. enterocolitica* were also recovered
145 by culturing. *Y. enterocolitica* of biotype 5 was the most common type found in 23 animals
146 from 3 farms. *Y. enterocolitica* of biotype 2 was found in 9 animals originating from 4 farms.
147 The in-herd prevalence of animals shedding biotype 5 was high (between 44 and 55%). Three
148 sheep shed also *ail*-positive *Y. enterocolitica* 1A in the feces.

149 **Table 2** Distribution of *ail*-positive *Yersinia enterocolitica* among 139 sheep originating from

150 11 farms.

Positive farms	Number of animals studied	Number of PCR-positive animals	Number of culture-positive animals	Number of animals with bioserotype		
				1A	2/O:9	5/O:3
I	10	1 (10 %)	1 (10 %)	1		
II	10	1 (10 %)	1 (10 %)		1	
III	10	1 (10 %)	0			
IV	10	4 (40 %)	1 (10 %)		1	
V	27	13 (48 %)	12 (44 %)			12
VI	7	1 (14 %)	0			
VII	20	8 (40 %)	7 (35 %)	2	5	
VIII	19	2 (11 %)	2 (11 %)		2	
IX	10	6 (60 %)	5 (50 %)			5
X	11	6 (55 %)	6 (55 %)			6
XI	5	2 (40 %)	0			
11 farms	139 animals	45 (32 %)	35 (25 %)	3	9	23

151

152 In total, 97 isolates showing bull's eye appearance on CIN agar plates were phenotypically

153 identified by API 20E and bio- and serotyped (Table 3). The colony morphology differed

154 clearly between the isolates of different biotypes. However, the colony morphology of *Y.*

155 *kristensenii* isolates on CIN resembled the colony morphology of the biotype 5 isolates.

Most (91/97, 94%) of the isolates were identified as *Y. enterocolitica*. However, biotype 5 isolates could not be identified as *Yersinia* spp. with API 20E. The obtained code (1015323) identified them as *Pantoea*. Only six (6%) of the 97 isolates were identified as *Y. kristensenii*. All *Y. enterocolitica* isolates of biotype 2 belonged to serotype O:9 (Table 3). All *Y. enterocolitica* isolates of biotype 5 were sorbitol and trehalose negative, and agglutinated with O:3 antiserum but only some (22%) of them agglutinated also with O:1,2 antiserum. Almost all isolates (99%) of biotypes 2 and 5 showed typical growth on CRMOX associated with the presence of the virulence plasmid. Biotype 1A isolates (18) were found from 8 animals. They formed blue colonies on the CHROMagar plates, thus differing from *Y. enterocolitica* isolates of biotypes 2 and 5, and *Y. kristensenii* isolates which formed mauve colonies.

Table 3 Phenotypic characteristics of 91 *Yersinia enterocolitica* and 6 *Yersinia kristensenii* isolates found in 40 sheep.

Yersinia spp.	Isolates		Bio-type	Sero-type	API 20E profile	CHROMagar Yersinia	CRMOX
Y. enterocolitica	5	(2)[a]	1A	O:8	1355723	Blue	-
	4	(3)	1A	O:5,8	1355723	Blue	-
	9	(3)	1A	NT	1355723	Blue	-
Y. enterocolitica	18	(9)	2	O:9	1155723	Mauve	+
	1	(1)	2	O:9	1155723	Mauve	-
Y. enterocolitica	12	(9)	5	O:1,2,3	1015323	Mauve	+
	42	(21)	5	O:3	1015323	Mauve	+
Y. kristensenii	6	(3)	NT	ND	1154703	Mauve	-

[a] Number of animals

NT=not typable, ND=not done

The *rfb* and *per* genes were detected by PCR in all serotype O:3 and O:9 isolates, respectively (Table 4). All isolates of biotype 5 carried the virulence genes in the chromosome (*ail*, *yst*A and *myf*A) and on the plasmid (*vir*F and *yad*A) which are typically detected only in pathogenic strains. One bioserotype 2/O:9 isolate was both *vir*F and *yad*A negative indicating that it does not carry the virulence plasmid. The same isolate also formed big and light colonies on CRMOX. Another isolate of the bioserotype 2/O:9 was both *yst*A and *myf*A negative. Almost all (16/17) biotype 1A isolates carried the *yst*B and *inv* genes but were *vir*F and *yad*A negative. Most of them were also *yst*A and *myf*A negative. Ten biotype 1A isolates from 3 sheep carried the *ail* gene usually detected only in pathogenic strains. Most (5/6) *Y. kristensenii* isolates carried the *yst*B gene and all 6 isolates were *vir*F and *yad*A negative.

Table 4 Virulence genes detected in 91 *Yersinia enterocolitica* and 6 *Yersinia kristensenii* isolates found in 40 sheep.

Yersinia spp.	Isolates		Number of isolates positive for								
			ail	*rfb*	*per*	*inv*	*ystA*	*ystB*	*myf*A	*virF*	*yadA*
Y. enterocolitica											
1A	18	(8)[a]	10	0	0	17	2	17	5	0	0
2/O:9	19	(9)	19	0	19	19	18	0	18	18	18
5/O:(1,2,)3	54	(23)	54	54	0	54	54	1	54	54	54
Y. kristensenii	6	(3)	0	0	0	1	0	5	0	0	0

[a] Number of animals

188 Forty *Y. enterocolitica* isolates were characterised with PFGE and their susceptibility to 14
189 antimicrobial agents were also tested. In total, 14 PFGE types were obtained with *Not*I and
190 *Xho*I enzymes (Table S2). Genetic diversity was very limited among the isolates belonging to
191 biotypes 2 and 5. Most (18/23, 78%) of the biotype 5 isolates were belonging to same PFGE
192 type and all isolates had the same *Not*I pattern. Six different *Not*I and *Xho*I patterns were
193 obtained among 8 biotype 1A isolates. No farm-specific PFGE types were found.
194
195 **Table S2** PFGE types obtained by *Not*I and *Xho*I enzymes found in 40 *Yersinia enterocolitica*
196 isolates from sheep originating from different farms.

Biotype	Number of isolates	Farm	PFGE type	*Not*I profile	*Xho*I profile
1A	2[a]	VII	I	N11	X11
	2	IV, VII	J	N12	X12
	1	IX	K	N13	X13
	1	I	L	N14	X14
	1	IV	M	N15	X15
	1	IV	N	N16	X16
2	4	II, VII, VIII	A	N21	X21
	3	IV, VII	B	N22	X22
	2	VII	C	N22	X22
5	18	V, IX, X	D	N51	X51
	2	V, X	E	N51	X52
	1	V	F	N51	X53
	1	V	G	N51	X54
	1	V	H	N51	X55

197

[a]One isolate per animal was characterised

All *Y. enterocolitica* isolates were susceptible to most (10/14) of the antimicrobial agents tested (Table S3). All isolates were resistant to ampicillin and some isolates showed resistance to ceftazidime. Resistance to trimethoprim and chloramphenicol occurred only among biotype 1A isolates (Table 5). No correlation between the PFGE and resistance patterns was observed. However, 3 biotype 5 isolates from sheep originating from the same farm having the same PFGE pattern showed resistance to ceftazidime.

Table S3 Minimal inhibitory concentration (MIC) of *Yersinia* isolates recovered from sheep feces obtained by VetMIC broth microdilution panel.

Antimicrobial agent	Break points	Biotype	Number of isolates with MIC (µg/µl)												
			0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
Ampicillin	EUCAST[a]	1A												2	4	2
		2													8	1
		5												8	15	
Cefotaxime	EUCAST[a]	1A				3	3	2								
		2				7		2								
		5		2	14	2	3	2								
Ceftazidime	EUCAST[a]	1A						1	1	4		1	1			
		2					2	4	1		2					
		5					13	4		5	1					
Chloramphenicol	EUCAST[a]	1A										7	1			
		2									9					
		5								23						
Ciprofloxacin	EUCAST[a]	1A		1	5	2										
		2	1		5	3										
		5	1	17	5											
Colistin	EUCAST[a]	1A						2	6							
		2							9							
		5						1	18	4						
Florfenicol		1A									3	5				
		2									9					
		5									23					
Gentamycin	EUCAST[a]	1A						1	6	1						
		2								9						
		5							19	4						
Kanamycin	CLSI[c]	1A										8				
		2										9				
		5										23				
Nalidixic acid	CLSI[c]	1A							8							
		2							9							
		5							23							
Streptomycin	EUCAST[b]	1A									5	3				
		2										9				
		5									10	13				
Sulfamethoxazole	EUCAST[b]	1A										7		1		
		2										9				
		5										22	1			
Tetracyclin	CLSI[c]	1A								6	2					
		2							1	6	2					
		5							21	2						
Trimetophrim	EUCAST[a]	1A						2	2	2	2	2				

	2 5			3 6	6 17			

[a] Breakpoints for Enterobacteriaceae

[b] Breakpoints for *Escherichia coli*

[c] No EUCAST or CLSI breakpoints available

Table 5 Distribution of PFGE types and resistance among 40 *Yersinia enterocolitica* isolates and one *Yersinia kristensenii* isolate.

Yersinia spp.	Isolates	Farms	PFGE types	Resistance to				
				Am	Caz	Cm	Su	Tm
Y. enterocolitica								
1A	6	I, IV, VI, VII,	I, J, L, M, N	R	S	S	S	S
	1	VII	I	R	R	S	S	I
	1	IX	K	R	R	R	S	I
2/O:9	7	II, IV, VII, VIII	A, B	R	S	S	S	S
	2	VII	C	R	I	S	S	S
5/O:(1,2,)3	17	V, IX, X	D, E, G, H	R	S	S	S	S
	3	V	D, E, F	R	I	S	S	S
	2	IX	D	R	I	S	S	S
	1	IX	D	R	R	S	S	S
Y. kristensenii	1	V	O	S	S	S	R	S

[a] Number of positive animals

Am=ampicillin, Su=sulfamethoxazole, Tm=trimethoprim, Cm=chloramphenicol, Caz=ceftazidime

DISCUSSION

All fecal samples from sheep at slaughter were screened for the possible occurrence of enteropathogenic *Yersinia* spp. using PCR to overcome the low sensitivity of cultural methods when healthy animals are studied (Fredriksson-Ahomaa & Korkeala, 2003). The PCR-prevalence of *ail*-positive *Y. enterocolitica* in fecal samples of young (< 1 year) sheep at slaughter was 11%. All older animals were negative for *Y. enterocolitica* and all animals for *Y. pseudotuberculosis.* This shows that young sheep can carry pathogenic *Y. enterocolitica* in the feces to the slaughterhouse and may serve as a contamination source of carcasses during slaughtering. In a recent study from Australia, fecal shedding of *Yersinia* spp. was studied in sheep on three occasions: during weaning, post-weaning and pre-slaughter using PCR (Yang et al., 2016). Fifteen percent of the lambs were shedding pathogenic *Y. enterocolitica* in the feces on at least one sampling occasion before slaughtering and the PCR-prevalence was highest just after weaning and lowest just before slaughtering.

Pathogenic *Y. enterocolitica* isolates carrying the pYV virulence plasmid were isolated from feces of 32 (8%) young sheep at slaughter: bioserotype 2/O:9 isolates were found in 9 animals and bioserotype 5/O:3 isolates in 23 animals. It is the first time that *Y. enterocolitica* bioserotype 5/O:3 was found in Finland. It is a rare bioserotype, which has mostly been isolated from wild hares in Europe and recently also in a wild alpine ibex in Switzerland (Joutsen et al., 2013; Le Guern et al., 2016). The hare type is typically agglutinated also with O:2 antiserum (Le Guern et al., 2016) but in our study only combined O:1,2 antiserum was commercially available, which may have influenced the results by giving weak-positive or false-negative results.

Bioserotype 2/O:9, which is a common finding in human yersinosis in Europe, has sporadically been isolated from ruminants, typically from cattle but also from sheep and goats

(Fredriksson-Ahomaa, 2015; Fredriksson-Ahomaa et al., 2012; Le Guern et al., 2016). However, bioserotype 2/O:9 has not been reported before in sheep in Finland. In Sweden, which is a brucellosis-free country, *Y. enterocolitica* 2/O:9 was recently isolated from feces of sheep serologically positive for *Brucella melitensis*. It has been shown that *Y. enterocolitica* 2/O:9 strains can give rise to false-positive results in serological tests for *B. melitensis* infection due to their identical O-antigen structure with *Brucella* spp. (Chenais et al., 2012).

Biotype 1A, which is considered a non-pathogenic biotype, was found in 8 young sheep. This is the most frequently found type in animal feces. In a recent study from China, *Y. enterocolitica* was found only in 1% of the 1677 sheep feces studied and all isolates were of biotype 1A (Liang et al., 2015). In Sweden, the prevalence of *Y. enterocolitica* in sheep was high; it was found in 35% of the slaughter sheep mostly in feces (Söderqvist et al., 2012). All isolates belonged to biotype 1A and were *ail* negative. In our study, surprisingly three young sheep shed *ail*-positive biotype 1A isolates in the feces. This gene is typically detected only in isolates belonging to pathogenic biotypes 1B and 2 to 5. However, there is growing evidence for *ail*-positive biotype 1A isolates (Fredriksson-Ahomaa et al., 2011, 2012; Kraushaar et al., 2011; Sihvonen et al., 2011; Liang et al., 2014). In China, the *ail* gene was detected in only two biotype 1A isolates (one was from a sheep) among 3870 isolates which shows that its occurrence is so far low in this biotype (Liang et al., 2014). However, it cannot be excluded that horizontal transfer of the *ail* gene may occur and increases the prevalence in non-pathogenic *Yersinia* spp.

Isolation and identification of biotype 2 and especially biotype 5 isolates was very challenging because of the slow growth and untypical colony morphology on CIN agar. Furthermore, API 20E could not identify biotype 5 isolates as *Yersinia* spp. Biotype 1A

isolates could easily be differentiated from biotypes 2 and 5 because they were growing well as blue colonies on CHROMagar and as big light colonies on CRMOX agar indicating that they are not carrying the virulence plasmid. *Y. kristensenii* isolates were difficult to differentiate from biotype 5 isolates on CIN agar because both were growing as very small and dark red colonies. *Y. kristensenii* forms also mauve colonies on CHROMagar like isolates of biotypes 2 and 5. However, on CRMOX agar they were growing as non-pathogenic isolates forming big and light colonies. The quite new commercial chromogenic agar used in this study has shown to be convenient to differentiate *Y. enterocolitica* 1A isolates from *Y. enterocolitica* isolates of biotypes 2 to 5 (Karhukorpi and Päivänurmi, 2013, Renaud et al., 2013). However, esculin-negative *Yersinia* spp. like in this study *Y. kristensenii* cannot be differentiated from *Y. enterocolitica* biotypes 2 to 5 (Karhukorpi and Päivänurmi, 2013).

All isolates of biotypes 2 and 5 carried *ail*, *yst*A and *myf*A genes in the chromosome which are generally present only in pathogenic *Y. enterocolitica* isolates (Fredriksson-Ahomaa, 2015; Schneeberger et al., 2015). Only one bioserotype 2/O:9 isolate did not carry the plasmid encoded genes *yad*A and *vir*F indicating that this isolate has lost the pYV virulence plasmid probably during the culturing. This isolate formed also big and light colonies on CRMOX agar at 37°C which is typical for pYV-negative isolates.

The commercial VetMIC GN-mo test panel based on broth microdilution was used in this study. It has also been used in some recent studies (Bonke et al., 2011, Schneeberger et al., 2015). All isolates of biotype 2 and 5 were very susceptible to the tested antimicrobials. The isolate were resistant only to ampicillin which is typical for most *Yersinia* isolates due to the *bla*A gene present in these isolates (Bonke et al., 2011). Only single isolates of the biotype 1A showed some resistance to ceftazidime, chloramphenicol and trimethoprim. Schneeberger et

296 al. (2015) studied bioserotype 4/O:3 isolates from humans and pigs with the same commercial
297 test panel and reported resistance to chloramphenicol, nalidixic acid, streptomycin and
298 sulfametoxazole in human isolates. Some differences in the susceptibility between different
299 bioserotypes may occur and could be one explanation. Another reason for the high
300 susceptibility of the sheep isolates may be the good animal health and the low use of
301 antimicrobials in sheep farming.
302
303 The PFGE analyses using two enzymes demonstrate that the genetic diversity of the biotype 2
304 and 5 isolates are very limited even though they differ clearly from each other. Most of the
305 types were circulating on different farms and they did not correlate with resistance patterns.
306 There was a high genetic diversity among biotype 1A isolates. Interestingly, two *ail*-positive
307 1A isolates from different sheep had the same unique genotype (I) and the third *ail*-positive
308 isolate from another farm had its own unique genotype (L). The resistance patterns did not
309 correlate with the genotypes either for biotype 1A isolates.
310
311 **Conclusions**
312 This study demonstrates that young healthy sheep can carry pathogenic *Y. enterocolitica* of
313 bioserotypes 2/O:9 and 5/O:3 which can be transmitted from the feces to the carcasses during
314 slaughtering. Isolation and identification especially of bioserotype 5/O:3 was shown to be
315 very challenging due to the slow growth and untypical colony morphology on the CIN plates,
316 and atypical phenotypic characteristics. The chromosomal encoded virulence gene *ail*, which
317 is typically only found in pathogenic *Y. enterocolitica* belonging to biotypes 1B and 2 to 5,
318 was detected in biotype 1A strains isolated from three young sheep originating from two
319 farms. The genetic diversity was very limited and antimicrobial susceptibility was very high
320 in biotype 2 and 5 isolates in contrast to biotype 1A isolates which were genetically

heterogeneous and showed some resistance to ceftazidime, chloramphenicol and trimethoprim.

Highlights

- *Y. enterocolitica* bioserotypes 2/O:9 and 5/O:3 were isolated from sheep < 1 year
- *ail*-positive *Y. enterocolitica* biotype 1A was found in three sheep
- Isolation and identification of bioserotype 5/O:3 is very challenging
- Genetic diversity of bioserotypes 2/O:9 and 5/O:3 was very limited
- Susceptibility to antimicrobial agents was very high

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References

Bhagat, N., & Virdi, J.S. (2007). Distribution of virulence-associated genes in *Yersinia enterocolitica* biovar 1A correlates with clonal groups and not the source of isolation. FEMS Microbiology Letters, 266, 177-18.

Bonke, R., Wacheck, S., Stüber, E., Meyer, C., Märtlbauer, E., & Fredriksson-Ahomaa, M. (2011). Antimicrobial susceptibility and distribution of β-lactamase A (*bla*A) and β-lactamase

345 B (*bla*B) genes in enteropathogenic *Yersinia* species. Microbial Drug Resistance, 17, 575-
346 581.
347
348 Chenais, E., Bagge, E., Thisted Lamberstz, S., & Artursson, K. (2012). *Yersinia enterocolitica*
349 serotype O:9 cultured from Swedish sheep showing serologically false-positive reactions for
350 *Brucella melitensis*. Infection Ecology and Epidemiology, 2, 19027.
351
352 CLSI (Clinical and Laboratory Standard Institute). (2008). Performance standards for
353 antimicrobial disk and dilution susceptibility test for bacterial isolated from animals; approves
354 standard. Third edition. M31-A3. Wayne, PA, USA.
355
356 CLSI (Clinical and Laboratory Standard Institute). (2014). Performance standards for
357 antimicrobial susceptibility testing. Twenty-fourth informational supplement. Clinical and
358 Laboratory Standards Institute. M100-S24. Wayne, PA, USA.
359
360 EFSA (European Food Safety Authority). (2015). The European Union summary report on
361 trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA
362 Journal, 13, 4329.
363
364 Fredriksson-Ahomaa, M. (2015). Enteropathogenic *Yersinia* spp. In Sing, A. (Ed.), Zoonoses-
365 Infections affecting humans and animals: focus on public health aspects (pp. 213-234).
366 Heidelberg: Springer.
367

368 Fredriksson-Ahomaa, M., & Korkeala, H. (2003). Low occurrence of pathogenic *Yersinia*
369 *enterocolitica* in clinical, food and environmental samples: a methodological problem.
370 Clinical Microbiology Reviews, 16, 220-229.
371
372 Fredriksson-Ahomaa, M., Cernela, N., Hächler, H. & Stephan, R. (2012). *Yersinia*
373 *enterocolitica* strains associated with human infections in Switzerland 2001-2010. European
374 Journal of Clinical Microbiology and Infectious Diseases, 31, 1543-1550.
375
376 Fredriksson-Ahomaa, M., Wacheck, S., Bonke, R., & Stephan, R. (2011). Different
377 enteropathogenic *Yersinia* strains found in wild boars and domestic pigs. Foodborne
378 Pathogens and Diseases, 6, 733-737.
379
380 Fukushima, H., Tsunomori, Y., & Seki, R. (2003). Duplex real-time SYBR Green PCR assay
381 for detection of 17 species of food- or waterborne pathogens in stools. Journal of Clinical
382 Microbiology, 41, 5134-5146.
383
384 Jacobsen, N. R., Bogdanovich, T., Skurnik, M., Lübeck, P. S., Ahrens, P., & Hoorfar, J.
385 (2005). A real-time PCR assay for the specific identification of serotype O:9 of *Yersinia*
386 *enterocolitica*. Journal of Microbiological Methods, 63, 151-156.
387
388 Joutsen, S., Sarno, E., Fredriksson-Ahomaa, M., Cernela, N., & Stephan, R. (2013).
389 Pathogenic *Yersinia enterocolitica* O:3 isolated from a hunted wild alpin ibex. Epidemiology
390 and Infection 141, 612-617.
391

392 Karhukorpi, J., & Päivänurmi, M. (2014). Differentiation of *Yersinia enterocolitica* biotype
393 1A from pathogenic *Yersinia enterocolitica* biotypes by detection of β-glucosidase activity:
394 comparison of two chromogenic culture media and Vitek2. Journal of Medical Microbiology,
395 63, 34-37.
396
397 Kraushaar, B., Dieckmann, R., Wittwer, M., Knabner, D., Konietzny, D., Mäde, D., &
398 Strauch, E. (2011). Characterization of a *Yersinia enterocolitica* biotype 1A strain harbouring
399 an *ail* gene. Journal of Applied Mircobiology, 111, 997-1005.
400
401 Le Guern, A-S., Martin, L., Savin, S., & Carniel, E. (2016). Yersiniosis in France: overview
402 and potential sources of infection. International Journal of Infectious Diseases, 46, 1-7.
403
404 Liang, J., Bi, Z., Shi, G., Xiao, Y., Qiu, H., Kou, Z., Hu, B., Jing, H., & Wang, X. (2014).
405 Two novel *ail*-positive biotype 1A strains of *Yersinia enterocolitica* isolated from China with
406 unequal adhesion and invasion properties. Infection, Genetics and Evolution, 27, 83-88.
407
408 Liang, J., Duan, R., Hao, Q., Yang, Y., Qiu, H., Shi, G., Gu, W., Wang, C., Wang, M., Tian,
409 K., Luo, L., Yang, M., Tian, H., Wang, J., Jing, H., & Wang, X. (2015). Ecology and
410 geographic distribution of *Yersinia enterocolitica* among livestock and wildlife in China.
411 Veterinary Microbiology, 178, 125-131.
412
413 Milnes, A.S., Stewart, I., Clifton-Hadley, F.A., Davies, R.H., Newell, D.G., Sayers, A.R.,
414 Cheasty, T., Cassar, C., Ridley, A., Cook, A.J.C., Evans, S.J., Teale, C.J., Smith, R.P.,
415 McNally, A., Toszeghy, M., Futter, R., Kay, A., & Paiba, G.A. (2008). Intestinal carriage of
416 verocytotoxigenic *Escherichia coli* O157, *Salmonella*, thermophilic *Campylobacter* and

417 *Yersinia enterocolitica*, in cattle, sheep and pigs at slaughter in Great Britain during 2003.
418 Epidemiology and Infection, 136, 739-751.
419
420 Renaud, N., Lecci, L., Courcol, R.J., Simonet, M., Gaillot, O. (2013). CHROMagar Yersinia,
421 a new chromogenic agar for screening of potentially pathogenic *Yersinia enterocolitica*
422 isolates in stool. Journal of Clinical Microbiology, 51, 1184-1187.
423
424 Rasmussen, H. N., Rasmussen, O. F., Andersen, J. K., Olsen, J. E. (1994). Specific detection
425 of pathogenic *Yersinia enterocolitica* by two-step PCR using hot-start and DMSO. Molecular
426 Cell Probes, 8, 99-108.
427
428 Schneeberger, M., Brodard, I., & Overesch, G. (2015). Virulence-associated gene pattern of
429 porcine and human *Yersinia enterocolitica* biotype 4 isolates. International Journal of Food
430 Microbiology, 198, 70-74.
431
432 Sihvonen, L. M., Hallanvuo, S., Haukka, K., Skurnik, M., & Siitonen, A. (2011). The *ail* gene
433 is present in some *Yersinia enterocolitica* biotype 1A strains. Foodborne Pathogens and
434 Diseases, 8, 455-457.
435
436 Slee, K.J., & Skilbeck, N.W. (1992). Epidemiology of *Yersinia pseudotuberculosis* and
437 *Yersinia enterocolitica* infections in sheep in Australia. Journal of Clinical Microbiology, 30,
438 712-715.
439

Söderqvist, K., Boqvist, S., Wauters, G., Vågsholm, I., & Thisted-Lambertz, S. (2012). *Yersinia enterocolitica* in sheep – a high frequency of biotype 1A. Acta Veterinaria Scandinavica, 54, 1-39.

Thisted Lambertz, S., Nilsson, C., Hallanvuo, S., & Lindblad, M. (2008a). Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. Applied Environmental Microbiology, 74, 6060-6067.

Thisted Lambertz, S., Nilsson, C., & Hallanvuo, S. (2008b). TaqMan-based real-time PCR method for detection of *Yersinia pseudotuberculosis* in food. Applied Environmental Microbiology, 74, 6465–6469.

Thoerner, P., Bin Kingombe, C. I., Bogli-Stuber, K., Bissig-Choisat, B., Wassenaar, T. M., Frey, J., & Jemmi, T. (2003). PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. Applied Environmental Microbiology, 69, 1810-1816.

Weynants, V., Jadot, V., Denoel, P., Tibor, A., & Letesson, J. J. (1996). Detection of *Yersinia enterocolitica* serogroup O:3 by a PCR method. Journal of Clinical Microbiology, 34, 1224-1227.

Wren, B. W., & Tabaqchali, S. (1990). Detection of pathogenic *Yersinia enterocolitica* by the polymerase chain reaction. Lancet, 336, 8716.

464 Yang, R., Ryan, U., Gardner, G., Carmichael, I., Campbell, A.J.D., & Jacobson, C. (2016).
465 Prevalence, fecal shedding and genetic characterization of *Yersinia* spp. in sheep across four
466 states of Australia. Australian Veterinary Journal, 94, 129-137.